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Prednison provokes serum and vasoactive substances in a mice model of immune thrombocytopenia

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ABSTRACT

Objective(s): The main objective of this study was to investigate the variations of β-endorphin (β-EP), vasoactive intestinal peptide (VIP), serotonin (5-HT) and norepinephrine (NE) of immune thrombocytopenia (ITP) mice as well as the regulatory mechanism of prednison.
Materials and Methods: Sixty BALB/c mice were randomly divided into control group, model group and prednison intervention group. ITP mice model was duplicated by injecting with glycoprotein-antiplatelet serum (GP-APS) except in control group. After ITP disease model was successful established, prednison was used in prednison intervention group. The β-EP, VIP, 5-HT and NE contents of ITP mice were detected by enzyme linked immunosorbent assay (ELISA).
Results: Compared with the values in control group, the detection values of VIP and 5-HT in model group declined, while the detection values of β-EP and NE increased. Compared with prednison intervention group, the detection values of VIP and 5-HT in model group increased, while the detection values of β-EP and NE showed no significant change.
Conclusion: In this study, the β-EP, VIP, 5-HT and NE contents in ITP mice injected with GP-APS were changed by prednison. It shows that prednison as the first-line therapy for ITP with effective hemostasis function is likely to increasing the contents of VIP and 5-HT. These results suggest the therapeutic value of prednison for the treatment of ITP.

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Introduction

It is generally believed that the pathogenesis of immune thrombocytopenia (ITP) is the abnormalities of cells and humoral immunity that lead to the generation of platelet antibodies (PAIgG PAlgM), and exert their impact on the platelet membrane glycoprotein, rendering the platelets in the reticuloendothelial system swallowed or destroyed. Such a series of changes decrease the platelet count, shorten the platelet life span and change the platelet function. In clinical practice, however, even though the platelet count of some ITP patients have not reached to safety level, but there is significant improvement in bleeding tendency after receiving adrenocorticotropic hormone therapy (1). It is thus speculated that its effector mechanism may also be closely related to vasoactive substances and neurotransmitters, such as β-endorphin (β-EP), vasoactive intestinal peptide (VIP), serotonin (5-HT) and norepinephrine (NE) apart from its effector mechanism of relieving bleeding and/or effective hemostasis (2) by reducing capillary permeability, inhibiting platelet antibodies, combating the destruction of antibody-absorbed platelet by macrophages and repairing platelet function. To verify this hypothesis, this research discusses the effective hemostatic mechanism of prednison against ITP by detecting the dynamic variations of platelet count and vasoactive substances after the establishment of ITP mice model using immune modeling method.

Materials and Methods

Animal

Fifty Hartley guinea pigs with the average weight of 250 g (half males and half females) were purchases from Tian Rui Experimental Animal Farm in Xing Ping City, Shan Xi province, China. Also, 140 BALB/c mice with the average weight of 18-22 g (half males and half females) were purchased from the Laboratory Animal Center of The Fourth Military Medical University.

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Reagent

Mouse β-EP, VIP, 5-HT, NE enzyme-linked immunosorbent assay (ELISA) kits were purchased from Wuhan Gene Biotech Co, LTD; complete Freund's adjuvant and incomplete Freund's adjuvant(Sigma-Aldrich, Made in the USA); enzyme-linked A protein(Boster Biology, made in China); prednison and P-nitrophenyl phosphate(PNPP) were provided by Shanghai Crystal PureBiological Technology Co, LTD; sodium azide was purchased from Zheng Zhou Pai Ni Chemical Reagent Factory; gelatin and polysorbate were purchased from Ke biological engineering co, LTD.

Apparatus

High speed micro-centrifuge (Chang Sha Xiang Yi H1650-W, made in China); high speed freezing centrifuge (Eppendorf 5804R, made in Germany); automatic animal blood analyzer (Perlong XFA6130, made in China); ELISA (Bio-Tek ELX800, made in the USA); water ultrafiltration system (Millipore Synergy UV, made in Germany).

Preparation of anti-platelet serum (GP-APS)

Anticoagulated whole blood taken from the anesthetized BALB/c mice was subject to gradient centrifugation to obtain platelet. The sample was washed 2 times with phosphate buffered saline (PBS), and then subjected to heavy suspension, counting, and then adjusting the concentration to 2.5 $\times 10^6$/ml. They were then evenly mixed with an equal amount of complete Freund's adjuvant and incomplete Freund's adjuvant as antigen. The antigen containing complete Freund's adjuvant was injected into guinea pigs' paws, back and subcutaneous areas at 0 week in a total of 5 points, for a total injection of 1 ml. The antigen containing incomplete Freund's adjuvant was injected into the same areas and same points at the same amount at the first, second and fourth week, respectively. Then, non-anticoagulated whole blood taken from the guinea pig's heart was centrifuged after standing for a while to obtain anti-platelet serum (GP-APS). The GP-APS was placed in a water bath at 56℃for 30 min to inactivate complement. The GP-APS was then diluted with saline to a concentration of 1: 4 to prevent erythrocyte adsorption (3).

Titer assay of anti-platelet serum (GP-APS)

The mouse platelets obtained from centrifugation was washed three times with PBS, and the concentration was adjusted to 6×10^4/μl. 50 μl platelets suspension was put into microtiter wells, which was centrifuged for 13 min at 560×g. Buffer was subsequently added (0.2% gelatin, 0.1% sodium azide, 0.05% polysorbate, 0.15 mol/l PBS), then incubated overnight at 4 ℃ and washed. 50 μl testserum was added, then incubated for 1 hr at 37 ℃ and washed. 100 μl enzyme-linked straphylococcus protein A was added, then incubated for 45 min at 22 ℃and washed for times. 200 μl PNPP (dissolved in the buffer of diethanolamine, with a concentration of 1.5 mg/ml) was added and incubated for 30 min at 37 ℃ Finally 50 μl sodium hydroxide was added to terminate the reaction. Optical density (absorbance value) was measured with a microplate reader at 405 nm wavelength (4).

Modeling grouping

The platelet count of blood taken from the tail vein of 60 BALB/c mice was detected with automatic animal blood analyzer. The 60 BALB/c mice were randomly divided into 3 groups of control group, model group and prednison group with 20 mice in each group, respectively. Mice in the control group were given intraperitoneal injection of 100 μl/20g saline; mice in the model group and prednison group was repeatedly given intraperitoneal injection of 100 μl / 20 g APS on a daily basis till the end of this experiment. On the eighth day of APS injection, the mice were given by gavage prednison liquid at a dose of 0.2 ml per 10 g body weight.

Detection of peripheral hemogram

The blood platelet (PLT), white blood cells (WBC) and hemoglobin (Hgb) of the proportionally diluted blood taken from the tail vein of the 60 mice were detected with automatic animal blood analyzer before APS injection and 8 days (starting administration of prednison), 12 days, and 15 days after APS injection.

Detection of vasoactive substances

After the experiment, the whole blood and eyeballs were taken from the anesthetized mice with the blood centrifuged after standing. The contents of β-EP, VIP, 5-HT, and NE of the collected serum were detected according to the manual of ELISA kits. Blank holes, standard holes and sample holes were respectively located in detection; Standards are diluted in the back plate of microtiter package to set the standard hole of concentration gradient with 50 μl/hole to be used for drawing of standard curve. 40 μl sample dilution was added into the sample holes followed by addition of 10 μl testing sample (that is 5-fold sample dilution), and then mixed well and incubated 30 min at 37 ℃ after closing plate. The plate was washed 5 times. 50 μl ELISA reagents was added into the holes except the blank hole, and mixed well and incubated for 30 min at 37 ℃ The plate was washed 5 times. 50 μl of color reagent was sequentially added into each hole, and mixed well and colorized in darkness for 15 min at 37 ℃ In addition, 50 μl of terminated solution was added into each hole to terminate the reaction. With the blank hole as zero, the absorbance values of each hole were

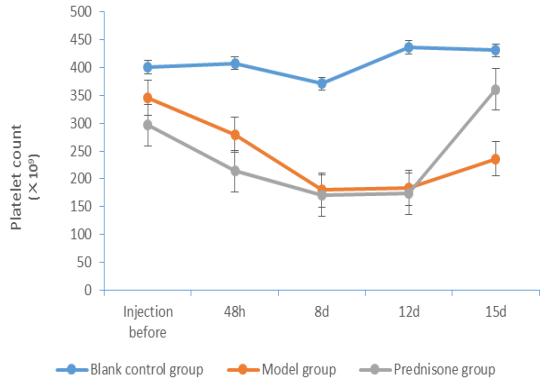

Figure 1. Dynamic variation of platelet count in BAL/c mice of Blank control group, model group, prednison group respectively, The count of platelet was detected by automatic animal blood analyzer for 15 days. Each point represents the average of platelet count in certain time point

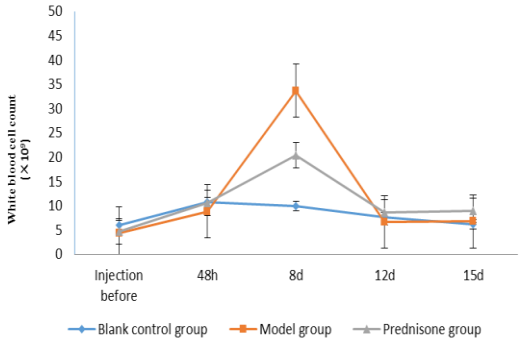

Figure 2. Dynamic variation of white blood cell count in BAL/c mice of Blank control group, model group, prednison group respectively, The count of white blood cell was measured with automatic animal blood analyzer for 15 days. Each point represents the average of platelet count of each groups in certain time point

sequentially measured by microplate reader at a wavelength of 450 nm within 15 min after the addition of terminated solution. The standard curve was drawn and the regression equation was established according to the concentration of the standard and corresponding OD value provided in the kit. The corresponding sample concentration was calculated on the regression equation according to OD values of samples.

Statistical methods

Experimental results were expressed with mean and standard deviation, and the comparisons among the groups were conducted using ANOVA. The pairwise comparisons were conducted using t test. It was considered statistically significant when $P<0.05$.

Results

APS titer assay

The results of APS titer assay showed that OD value (405 nm) in APS serum group (0.300±0.037) was twice as much as that in the control group (0.163±0.012)

Blood detection

Blood detection results of the mice in different groups were shown in Figure 1 to 3. It can be concluded from Figure 1that before APS injection, comparisons of platelet count among different groups were not statistically significant ($P>0.05$). Also, 48 hr after APS injection, the platelet count of mice in model group and prednison group declined, and comparison of their count with that in control group was statistically significant ($P<0.05$). During the period between the 8th day and the 12th day after APS injection, the platelet count in model group and prednison group declined to the lowest point and comparison of them with that in control group was found to be statistically significant ($P<0.01$). On the 15th day following APS injection, compared with that in control group, platelet count in model group still remained at a low level ($P<0.01$); while the platelet count in prednison group (that is the 8th day after the administration of prednison) increased significantly, and comparison of the platelet count with that in model group was statistically significant ($P<0.01$).

It can be concluded from Figure 2 that before and 48 hr after APS injection, comparisons of WBC count among different groups were not statistically significant ($P>0.05$). On the 8th day following APS injection, the WBC count in model group and prednison group increased significantly compared to that in control group. This may be due to the immune response caused by APS injection. During the period between the 12th day and 15th day after APS injection, the WBC count in each group returned to normal. A comparison of WBC count with that in control group was not statistically significant ($P>0.05$).

It can be concluded from Figure 3 that before APS injection, hemoglobin values among different groups compared with control group were not statistically significant ($P>0.05$). During the period between 48 hr and 12th day following APS injection, the hemoglobin values in all groups declined. Comparison of the results with that in control group was statistically significant ($P<0.05$). The hemoglobin value in prednison group increased on the 15th day (that is, the 8th day after the administration of prednison). Comparison of the results with that in model group was statistically significant ($P<0.05$).

Detection of vasoactive substances

The serum of mice in each group was collected after the experiment finished. The contents of β-EP, VIP, 5-HT and NE were measured according to the manual of ELISA kits. The detection results were shown in Figure 4.

It can be concluded from Figure 4 that β-EP the content of β-EP in model group increased in comparison with that in control group ($P<0.05$); moreover, compared to that in model control group,

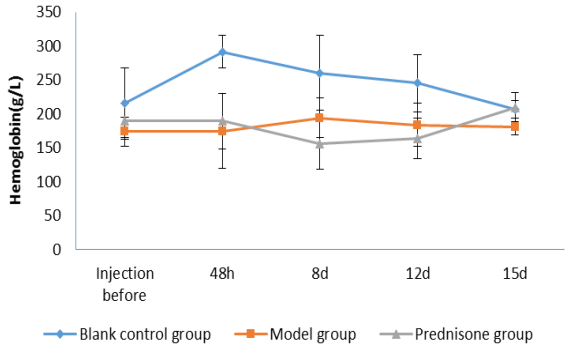

Figure 3. Dynamic variation of hemoglobin content in BAL/c mice of Blank control group, model group, prednison group respectively, The hemoglobin content was measured with automatic animal blood analyzer for 15 days. Each point represents the average of hemoglobin content in certain time point

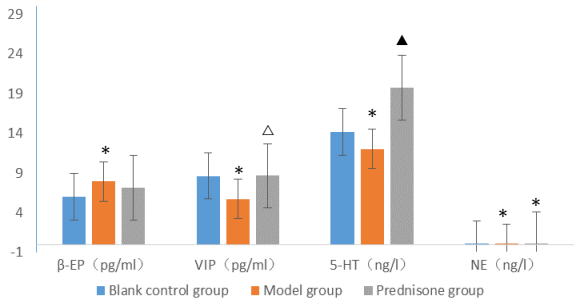

Figure 4. Optical density of β-EP, VIP, 5-HT, NE in all groups of BALB/c mice were measured by method of ELISA after last booster, β-EP, VIP, 5-HT, NE, each bar represents the mean± SD. Compared with the blank control group, *p<0.05; Compared with the model group, △*P*<0.05, ▲*P*<0.01

the content of β-EP did not decline even after prednison intervention (*P*>0.05). Also, compared with that in control group, the content of VIP declined (*P*<0.05); in addition, compared to model control group, the content of VIP increased after prednison intervention (*P*<0.05). Furthermore, compared with control group, the content of 5-HT declined in model group (*P*<0.05). Also, compared with that in model control group, the content of 5-HT increased after prednison intervention (*P*<0.01). Compared with that in control group, the content of NE increased in model and prednison group (*P*<0.05).

Discussion

With ITP as the representative, hematological autoimmune diseases are characterized by reduction of peripheral platelet count coupled with spontaneous bleeding of skin and mucous membrane. It is believed the pathogenesis of ITP is due to abnormal function of cellular and humoral immunity, which lead to the generation of anti-platelet antibodies, rendering the platelet massively swallowed. The number of platelets thus decreased and the quality lowered as well (5).

Mouse platelets injecting into guinea pigs induce antigen-antibody reaction and produce GP-APS in serum. This kind of serum with GP-APS injected into mouse has resulted in coating that mouse platelets with GP-APS, which are removed from the circulation by the fixed phagocyte system. In our study, mouse platelets as antigen injected into guinea pigs to induce GP-APS. Guinea pigs' swelling on the injection sites was showed in our study. It is caused by antigen-antibody reaction in guinea pigs. Considering the efficiency of anti-platelet serum and safety of guinea pigs, 5 weeks immunizing process was performed to prepare the anti-platelet serum in guinea pigs by injected mouse platelets. With anti-platelet serum injected into mice, the platelet count declined in model and prednison group, and maintained at lower level than normal group. It means that GP-APS of guinea pigs mediated platelets destruction in mouse. This passive immunization is a suitable method to imitate the pathological mechanism of ITP. The WBC count also increased and hemoglobin decreased in model and prednison group during the modeling process. The results demonstrate that GP-APS injected into mice could simulate process of ITP in human.

As the first-line therapy for ITP, principal mechanism of prednison involves suppressing humoral and cell-mediated immune response that reduces the binding ability of the immunoglobulin to cell surface receptors, and inhibits the synthesis and release of interleukin. Hence, it reduces the transformation of T lymphocytes to lymphoblastoid cell and mitigates the expansion of primary immune response. Also, prednison can boost function of preventing immune complexes from passing through the basement membrane, reducing the complement components and immunoglobulins concentration. Moreover, prednison helps to reduce the perme-ability of capillary wall and the cell membrane; therefore, prednison may inhibit inflammatory exudation and reduce the incidence of skin purpura. According to clinical observations, the improvement of bleeding tendency is not proportionate to the platelet counts rise after the administration of prednison, suggesting that prednison does not simply stop bleeding through immune regulation or enhancing platelet value. Therefore, it is likely an unnoticed hemostatic mechanism involving capillary function and its vasoactive substances or neuro-transmitters.

At the end of our study, platelet count of prednison group was increased compared with model group. This demonstrates the efficiency of prednison in ITP. The WBC count was normal, but hemoglobin was increased after treatment with prednison. The results showed that the mechanism of prednison in treating ITP is related to regulated immune homeostasis and function of hemostasis. Findings also showed that platelet count of prednison group maintained higher than other groups till 15th day, while WBC count decreased to normal level from 12th day, and hemoglobin was not

decreased significantly. These results showed that other factors were involved in prednison treating ITP.

Based on human physiological features, three factors including platelets, vascular and coagulation factor are involved in normal hemostatic mechanism. Also, capillary wall function, intravascular sub-cutaneous collagen and vasoconstriction play important roles in hemostasis, and the normal functioning of capillary requires the regulation of vasoactive substances and neurotransmitters such as β-EP, VIP, 5-HT and NE.

As an important neuropeptide, β-EP has opiate-like effects, which plays various important roles in regulating respiratory, circulatory and digestive functions as well as regulating the functions of thalamus, pituitary and adrenal axis. It is also involved in pathological processes of shock, stroke, analgesia (pain), headache, depression, epilepsy, and brain and spinal cord injury (6-10). Besides, β-EP is widely distributed in the central nervous system and the outer periphery systems (11).

Some researches already confirmed that VIP is related to the occurrence, development, outcome, treatment and prognosis of many diseases. It is especially closely related to diseases such as gastrointestinal motility disorders, vascular function activity, chronic obstructive pulmonary disease, and liver and gallbladder diseases (12-15). Vasoactive intestinal peptide helps to relax smooth muscles by promoting the synthesis of nitric oxide (NO) by target cells. Its main function involves dilation of blood vessels, transmission of information, and regulating immunity.

In peripheral tissues, 5-HT is a strong vasoconstrictor and smooth muscle contraction stimulants (16). More than 95 percent of the 5-HT is stored in dense granules of platelet whose activation releases 5-HT that is involved in stopping the bleeding or the formation of blood clots. Therefore, the variation of 5-HT is closely related to the platelet function. In the immune regulation network, 5-HT also plays regulatory role of immune cells and immune molecules. Therefore, 5-HT may share a common pathway with the pathogenesis of ITP.

As another neurotransmitter, NE is mainly secreted from adrenal medulla. Through binding to the receptor on vascular smooth muscle cell membrane, norepinephrine (NE) contributes to vasoconstriction of the blood vessels of the skin, mucous membranes and internal organs such as kidneys. It is currently used for the treatment of shock (17-18). But, it is conducive to hemostasis in case of bleeding.

Our study showed the changes of β-EP, VIP, 5-HT and NE among control group, model group and prednison group. The results support that vasoactive substances and neurotransmitters are not just important components of human immune system, but also are likely to be one of the key mechanisms involved in bleeding and hemostasis. Therefore, discussion of the variations of vasoactive substances of ITP mice is conducive to the elaboration of ITP bleeding and the regulatory mechanism of prednison.

Conclusion

Based on the findings of this research, it can be concluded that the duplication of ITP mice by APS injection immunization modeling method is similar to human incidence of ITP whose pathogenesis not only directly involves immune response, but also is related to the variations of β-EP, VIP, 5-HT and NE contents. Also, as the first-line therapy for ITP, prednison boost the function of hemostatic effect. Increasing the contents of VIP and 5-HT is also one of its effector mechanisms apart from immunoregulatory function as well as increasing and improving the blood platelet value and function. The discovery of new regulatory mechanism of prednison in the treatment of ITP provides an important basis for the short-term impact of therapy involving the use of high-dose adrenocortical hormone for the ITP patients whose peripheral blood platelet count are in a non-safety level and are suffering the risk of potential visceral bleeding.

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